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The role of upstream U3 sequences in HIV-1 replication and CD4$^+$ T cell depletion in human lymphoid tissue ex vivo

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Abstract

The LTRs of all primate lentiviruses contain long U3 regions overlapping the *nef* gene. To assess the relevance of the modulatory U3 region for HIV-1 replication, we inactivated the T-rich region, the Polypurine tract and attachment (*att*) sequences in *nef* by silent mutations and inserted intact *cis*-regulatory elements just upstream of the core enhancer. These modifications severely truncated the U3 region and eliminated the *nef* overlap. The resulting HIV-1 mutants expressed functional Nef, replicated efficiently and caused CD4$^+$ T cell depletion in ex vivo-infected lymphoid tissue suggesting that the modulatory U3 region might not be essential for efficient HIV-1 gene expression and AIDS pathogenesis.

Keywords: HIV-1; Polypurine tract; LTR; Nef; U3 region

Introduction

Retroviral genomes are flanked by the long terminal repeats (LTRs), which are divided in three regions: U3, R and U5 (Gaynor, 1992; Varmus, 1988). The U3 region of human and simian immunodeficiency viruses (HIV and SIV, respectively) contains the basal promoter (nt −78 to −1), a core enhancer (nt −105 to −79) and a very long modulatory region (nt −454 to −104) (Gaynor, 1992). The three consensus SP1 binding sites in the basal promoter and the two NF-kB binding sites in the core enhancer are the major elements involved in the regulation of HIV-1 transcription (Gaynor, 1992). Numerous studies also proposed, however, that the modulatory U3 region influences viral gene expression. Early reports indicated that this region might contain a negative regulatory element (Rosen et al., 1985; Siekevitz et al., 1987). A large number of cellular factors including NF-AT, Ets-1, USF, AP-1, COUP and Sp1 have been proposed to interact with the modulatory region and to contribute to HIV-1 LTR promoter activity (Pereira et al., 2000). However, the modulatory U3 region is also entirely overlapped by the *nef* gene (Kirchhoff et al., 1995). Studies with SIV indicate that the upstream U3 sequences (herein after referred to as US region) might serve mainly as a *nef* coding sequence. Initially, it has been shown that additional deletions in the modulatory U3 region emerge in rhesus macaques infected with a *nef*-deleted SIVmac mutant (Kirchhoff et al., 1994). A subsequent study demonstrated that nucleotide changes in the US region, which did not alter the Nef coding sequence, did not attenuate the virulence of SIVmac (Ilyinskii et al., 1994). More recently, we have shown that an SIV variant containing a grossly truncated U3 region that does not overlap the *nef* gene replicated efficiently and caused disease in infected macaques (Münch et al., 2001).

These previous studies demonstrated that the conserved overlap between *nef* and the U3 region is not obligatory for efficient replication and pathogenicity of SIV. Findings from HIV-1-infected long-term survivors demonstrate that the HIV-1 US region is also only preserved in the

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presence of an intact *nef* open reading frame (Deacon et al., 1995; Kirchhoff et al., 1995; Rhodes et al., 2000; Salvi et al., 1998). However, these HIV-1 variants were strongly attenuated and it remained elusive whether the US region might contribute to viral replication and pathogenicity in the presence of an intact *nef* gene. We have previously mutated the critical *cis*-regulatory elements, encompassing the T-rich region, polypurine tract and attachment (att) sequences required for integration (herein after referred to as TPI region) in the SIVmac239 *nef* gene (Münch et al., 2001). The resulting SIV mutants had a short U3 region which did not overlap the *nef* gene. Thus, they allow to study Nef function in the context of replication-competent viruses without the complications of overlapping LTR sequences. We also demonstrated that the modulatory U3 region is not required for a virulent phenotype in infected macaques (Münch et al., 2001). The sequence homology between HIV-1 and SIVmac Nef proteins is limited and several activities are mediated by different domains. Some functions, such as down-modulation of CD3 and CD28, are only efficiently carried out by SIVmac but not by HIV-1 Nef proteins (Schindler et al., 2004). Thus, some observations made with SIVmac Nef variants might not be relevant for HIV-1. Furthermore, although a large number of interacting cellular factors have been described (Pereira et al., 2000), the role of the HIV-1 modulatory U3 region for LTR-directed transcription and viral replication is less clear than that of SIVmac. In the present study, we generated HIV-1 variants without the overlap between *nef* and U3 sequences. Our goals were to assess the relevance of the modulatory U3 region for viral replication and cytopathicity and to establish an experimental system allowing to study HIV-1 Nef function independently of overlapping LTR and critical *cis*-regulatory sequences.

Results and discussion

A total of 12 point mutations were introduced into the *nef* gene to inactivate the *cis*-regulatory TPI element (Fig. 1A). These changes did not alter the predicted Nef amino acid sequence. Full-length or prematurely truncated wild type (wt) and TPI mutant *nef* alleles were inserted into proviral NL4-3 constructs containing a 222-bp deletion in the *nef* unique region alone (Δ1) or in conjunction with a 266-bp U3 deletion (Δ1Δ2) (Fig. 1B). The 3′ ends of the mutant forms of NL4-3 generated are shown in Fig. 1B. TPImut differs from NL4-3wt only by the specific mutations in the TPI region. The Δ1-derived constructs contain intact or mutated *nef* genes upstream of the full-length LTR resulting in a duplication of the U3 region and an enlarged viral genome. In comparison, the Δ1Δ2-derived NL4-3 mutants contain just the intact U3 sequences in the *nef* gene and 83-bp of the original sequence upstream of the NF-κB sites. The right panel of Fig. 1B shows the 5′LTRs predicted after reverse transcription. The TPImut NL4-3 variant is predicted to be inactive because it lacks sequences critical for reverse transcription and integration. In contrast, the *nef*+Δ1 and *nef*+Δ1Δ2 HIV-1 and otherwise isogenic forms containing two premature stop signal at the 72 and 73 codons of the *nef* gene (*nef**) contain two copies of the TPI elements, which might result in two different forms of the proviral 5′LTR. Finally, the *nef*TPImutΔ1 mutant is predicted to contain the full-length U3 region at the 5′ end of the genome, whereas the U3 region of *nef*TPImutΔ1Δ2 is predicted to be grossly truncated. The HIV-1 NL4-3 *nef*TPImutΔ1Δ2 genome contains U3 regions that are 266-bp shorter than the 454-bp NL4-3wt U3 region. Nonetheless, it contains all known coding sequences and *cis*-acting elements. In addition to the mutants shown in Fig. 1B, forms containing disrupted *nef* genes (*nef**) or just the five mutations localized in the polypurine tract (Pmut), were also generated. Furthermore, we constructed otherwise isogenic forms containing the 005pf135 V3 region (Papkalla et al., 2002) in the viral envelope to generate CCR5-tropic HIV-1 NL4-3 variants.

To investigate the infectivity and replicative potential of the HIV-1 TPI mutants, virus stocks were generated by transient transfection of 293T cells as described previously (Rucker et al., 2004). With the exception of NL4-3 TPImut and Pmut, all mutant viruses replicated efficiently in Jurkat T and CEM × 174 5.25 M7 cells. Genomic DNA was extracted at the end of culture for PCR and sequence analysis. PCR amplification with primer p1, which binds to the US region, paired with p2 binding to the non-translated region downstream of the 5′LTR and upstream of *gag*, yielded fragments of 655-bp and 761-bp for the NL4-3wt- and *nef*+Δ1Δ2-infected cells, respectively (Fig. 1C). The size of the *nef*+Δ1Δ2-derived fragment is enlarged by 106-bp due to the insertion of a fragment encompassing the T-rich region, the polypurine tract and 83-bp of upstream U3 sequences. Consistent with the absence of the US region at the 5′ end of the proviral genome (Fig. 1B), no PCR fragment was obtained from DNA extracted from *nef*TPImutΔ1Δ2-infected cells (Fig. 1C, lane 4). In contrast, PCR amplification of p1 paired with p4, which binds in the U5 region (Fig. 1B), yielded amplification products of the predicted size (541-bp and 646-bp, respectively) for all three infected cell cultures (Fig. 1C, lanes 7–9). The differences in amplification efficiency are in agreement with the fact that p1 binds to both LTRs of the NL4-3wt and *nef*+Δ1Δ2 proviruses but only to the 3′LTR of the *nef*TPImutΔ1Δ2 proviral sequence. Predictably, amplification with p1 and p5, which binds to the 5′ end of the U3 region, resulted in amplification products of 344-bp for cells infected with the NL4-3 mutant viruses (Fig. 1C, lanes 13 and 14). In contrast, no specific PCR product was obtained with DNA extracted from NL4-3wt-infected cells because 3′ primer (p5) binds only upstream of the 5′ primer (p1) in the wild type LTR (Fig. 1B). In sum, PCR amplification and sequence analysis of

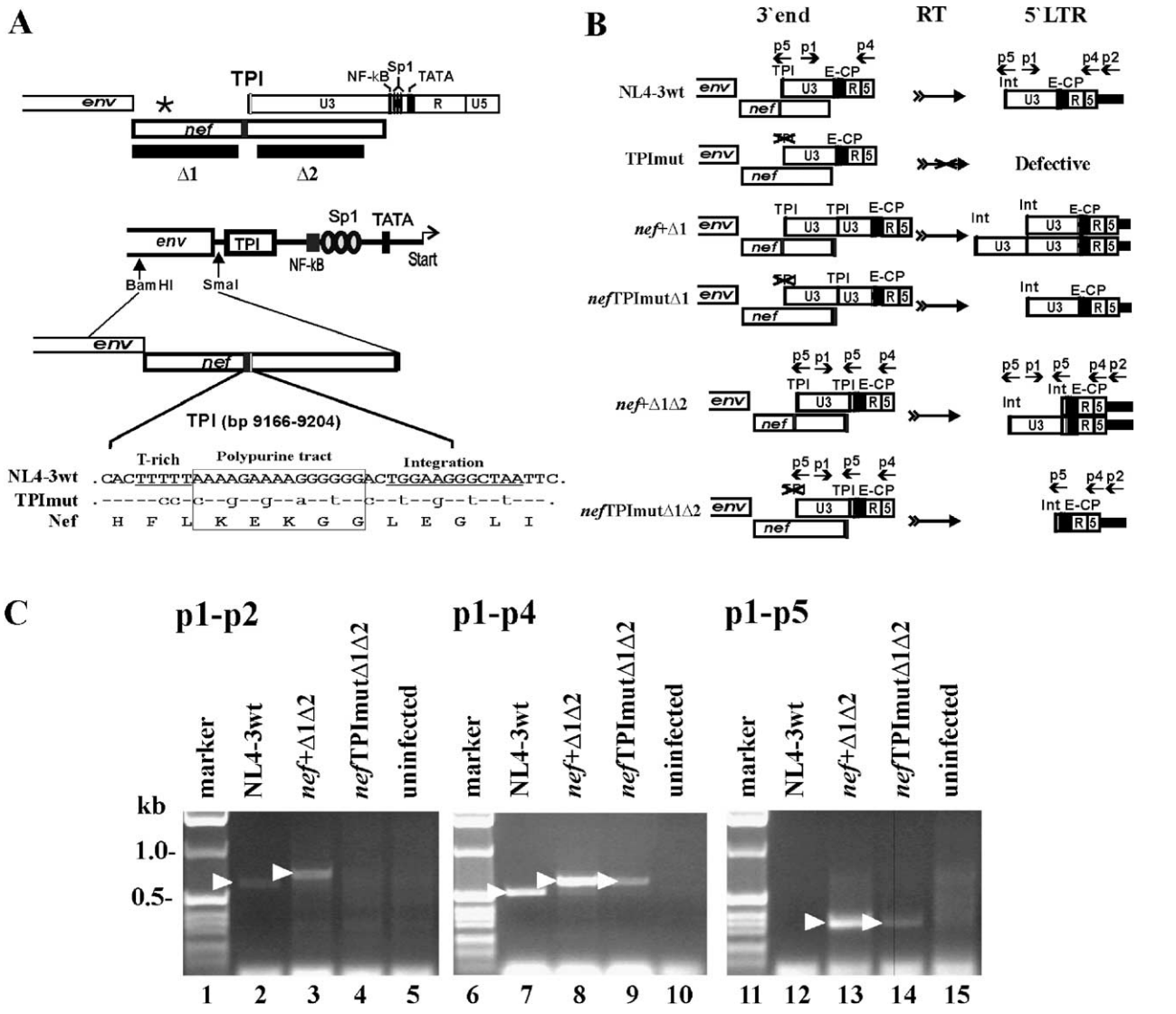

Fig. 1. Mutant construction and HIV-1 NL4-3 *nef*/LTR variants analyzed. (A) NL4-3 constructs containing a deletion of 222-bp in the *nef* unique region alone (Δ1) or together with a deletion of 266-bp in the U3 region (Δ1Δ2) (Gibbs et al., 1994), were utilized to generate *nef*/LTR variants. The black bar indicates the position of the deletions. As indicated below, 12 (TPImut) or 5 (Pmut) silent point mutations were introduced in the TPI-region and cloned into NL4-3wt (TPImut, Pmut) or the Δ1 (*nef*TPImutΔ1, *nef*PmutΔ1) and Δ1Δ2 (*nef*TPImutΔ1Δ2, *nef*PmutΔ1Δ2) proviral constructs. (B) Schematic presentation of the HIV-1 NL4-3 constructs analyzed (left), and the deduced 5′LTR promoter region (right). All mutants shown contain intact *nef* genes. TPImut differs from NL4-3wt only by the specific point mutations in the TPI region; *nef*+Δ1 and *nef*+Δ1Δ2 contain NL4-3wt *nef* genes; *nef*TPImutΔ1 and *nef*TPImutΔ1Δ2 TPI-mutated *nef* alleles. Otherwise, isogenic forms with a premature stop signal in the *nef* ORF were also generated. The Δ1Δ2 forms contain only 83-bp of US sequences. Abbreviations: E-CP, enhancer-core promoter; Int, U3-terminal sequences required for integration. Arrows indicate the positions of primers used for PCR amplification. (C) HIV-1 5′LTR and *nef*-3′LTR sequences were amplified from genomic DNA extracted from infected CEM × 174 5.25 M7 cells. The arrowheads indicate DNA fragments of the expected size. Similar results were obtained with infected Jurkat T cells.

nef/LTR and LTR/*gag* sequences at the end of culture demonstrated that all proviruses contained the predicted U3 sequences at the 5′ and 3′ ends of their genomes.

Western blot analysis showed that HIV-1 variants containing NL4-3wt or TPI-mutated *nef* genes, but not the *nef** variants, expressed normal quantities of full-length Nef proteins (Fig. 2 and data not shown). As expected from previous studies (Papkalla et al., 2002), intact *nef* genes strongly enhanced infectivity of both R5- and X4-tropic HIV-1 NL4-3 variants (Fig. 3). The TPImut variant missing critical *cis*-regulatory elements was grossly defective. In contrast, the infectivity of the *nef*+Δ1Δ2 and *nef*TPImutΔ1Δ2 variants, containing intact TPI sequences downstream of *nef* and upstream of the core enhancer, was only moderately reduced compared to NL43wt (Fig. 3). Similar results were obtained with the Pmut forms and the Δ1-derived constructs (data not shown). These results demonstrate that the TPI-mutated *nef* alleles enhance HIV-1 infectivity and that the presence of the full-length modulatory U3 region at the 5′ end of the viral genome moderately enhances viral gene expression and/or infectivity.

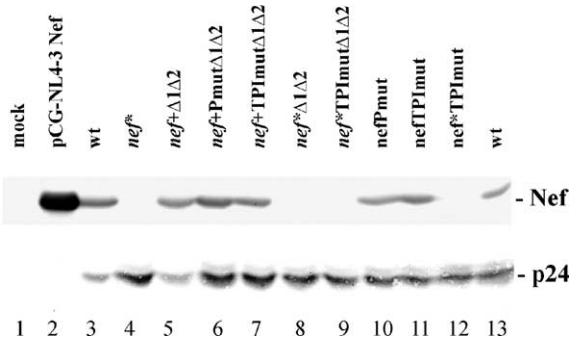

Fig. 2. Mutations in the TPI region do not impair HIV-1 Nef expression. Nef and p24 core antigen expression in 293T cells transiently transfected with the indicated proviral NL4-3 constructs was verified by immunoblot using rabbit-anti-HIV-p24 serum provided by the AIDS Research and Reference Reagent Program or an anti-HIV-1 Nef mAb directed against amino acids 151 to 170 (Advanced Biotechnologies). As a positive control, cells were transfected with the pCG-NL4-3Nef vector, which contains the CMV promoter and coexpresses Nef and the green fluorescent protein (lane 2). Similar results were obtained in an independent experiment.

Next, we investigated the replicative capacity of the HIV-1 NL4-3 TPI variants in human peripheral blood mononuclear cells (PBMC). All NL4-3 variants, except the TPImut and Pmut forms, replicated in pre-stimulated PBMC, although intact *nef* genes resulted in moderately increased replication efficiency (Fig. 4A). As expected (Chowers et al., 1994; Miller et al., 1994; Spina et al., 1994), Nef increased HIV-1 replication more strongly when PBMC were infected immediately after isolation and stimulated 3 days later (Fig. 4B). The Δ1Δ2-derived constructs containing the wt, Pmut or TPImut mutant *nef* alleles replicated with NL4-3wt-like efficiency in the PBMC cultures, although the replication kinetics were slightly to moderately delayed compared to NL4-3wt in PBMC derived from some blood donors (data not shown). Thus, these constructs allow to assess the effect of HIV-1 *nef* on viral infectivity and replication without the restriction of overlapping LTR and *cis*-regulatory sequences. Further analysis showed that amino acid changes in the region of Nef that is encoded by the TPI-region impair Nef protein stability and reduce HIV-1 infectivity and replication (data not shown). Thus, this region in Nef is conserved because it contains critical *cis*-regulatory elements and is important for stable Nef expression.

The genomes of all primate lentiviruses are organized so that *nef* overlaps the 3′LTR suggesting that this genomic organization is advantageous in the infected host. We have previously shown, however, that an SIVmac239 TPI variant containing a U3 region that is shortened by 384-bp and does not overlap *nef* is pathogenic in infected rhesus macaques (Münch et al., 2001). The homology between the HIV-1 and SIV U3 LTR sequences is low and it has been demonstrated that the SIVmac U3 region shows a higher degree of functional redundancy compared to the corresponding HIV-1 region (Ilyinskii and Desrosiers, 1998; Pöhlmann et al., 1998). To further evaluate the possible relevance of the modulatory U3 region for HIV-1 replication and pathogenicity in infected humans, we infected ex vivo tonsillary human lymphoid tissue (HLT) with the HIV-1 *nef*/LTR variants. This experimental system is likely of high physiological relevance because it does not require exogenous stimulation and the complexity of cell types mimics the in vivo situation (Glushakova et al., 1995; Glushakova et al., 1999). Mutations in the TPI region (*nef*+TPImut, *nef**TPImut) disrupted the replicative capacity of HIV-1 (Fig. 5A). Insertion of an intact TPI region upstream of the core enhancer (*nef*+TPImut) restored the ability of both X4- and R5-tropic HIV-1 variants to spread efficiently in ex vivo-infected HLT. Similarly to the results obtained using human PBMC, however, the mutant viruses replicated with delayed kinetics in tissues derived from some donors (example shown in Fig. 5A, lower panel). Nevertheless, these results demonstrate that elimination of the *nef*-LTR overlap and truncation of the U3 region does not have major disruptive effects on the ability of HIV-1 to replicate in

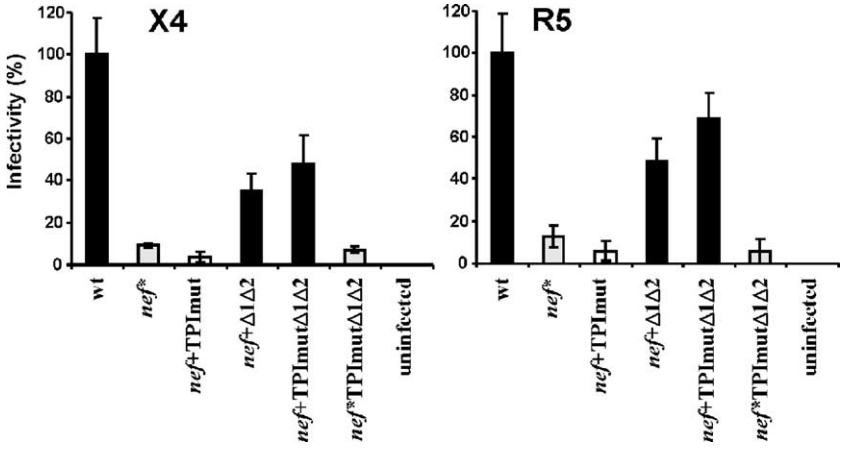

Fig. 3. HIV-1 *nef* alleles containing changes in the TPI-region enhance viral infectivity. P4-CCR5 cells were infected with the indicated X4-tropic (left) or R5-tropic (right) HIV-1 NL4-3 LTR/*nef* variants. Infections were performed in triplicate with virus stocks of the X4- or R5-tropic HIV-1 NL4-3 variants containing 3.0 ng p24 antigen. Shown are average values ± SD. Infectivity is shown relative to HIV-1 NL4-3wt. Similar results were obtained in two independent experiments with different virus stocks.

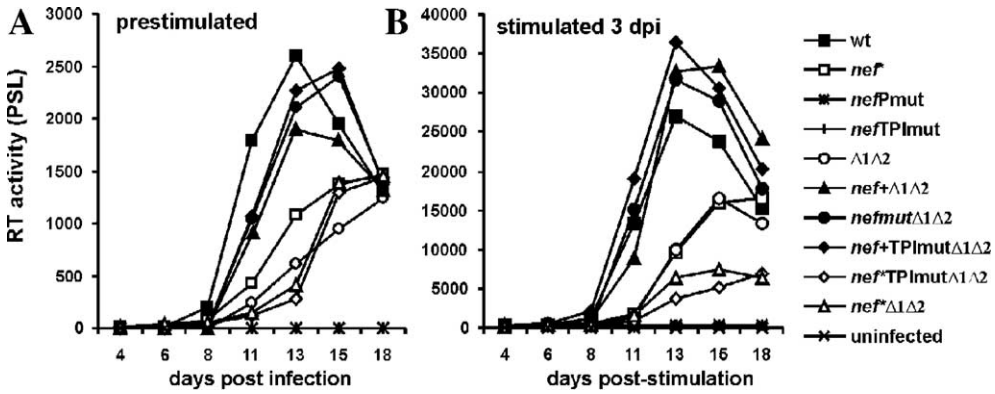

Fig. 4. HIV-1 NL4-3 variants without overlapping *nef*/U3 sequences replicate efficiently in human PBMC. (A) PBMC were PHA/IL-2-stimulated for 3 days prior to infection. (B) Unstimulated PBMC were infected with X4-tropic HIV-1 *nef*/LTR variants immediately after isolation, stimulated with PHA/IL-2 for 48 h 3 days later and cultured for 18 days. Virus production was monitored by reverse transcriptase assay as described (Potts, 1990). Similar results were obtained in independent experiments using different virus stocks and PBMC from different donors. PSL, photon-stimulated light emission.

lymphoid tissue. Concordant with the different kinetics of replication, the X4-tropic form of the *nef*+TPImutΔ1Δ2 mutant virus depleted CD4$^+$ T cells as effectively as HIV-1

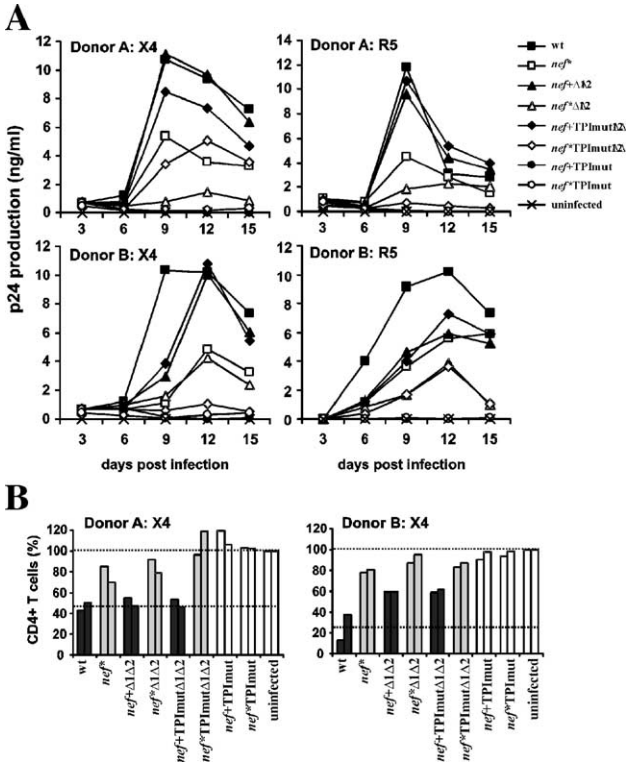

Fig. 5. Effect of a truncated U3 region on HIV-1 replication and CD4$^+$ T cell depletion in HLT. (A) Replication of the indicated X4-tropic (left) or R5-tropic (right) HIV-1 NL4-3 Nef variants in ex vivo-infected HLT derived from two donors. Infections were performed without adding exogenous IL-2 with virus stocks containing 10 ng of p24 antigen and virus production was measured by p24 ELISA at the indicated days post-infection. (B) Percentages of CD4$^+$ T cells in the tissue blocks (left bars) and cells that migrated in the gel foams (right bars) at the end of culture at 15 days post-infection. Tissue culture, infections and FACS analysis were performed as described previously (Rucker et al., 2004). NL4-3wt-like and moderately delayed replication kinetics, respectively, were also observed in two independent experiments using tissues derived from different donors.

NL4-3wt in tissues derived from donor A (Fig. 5B, left panel) but was partially attenuated in HLT derived from donor B (Fig. 5B, right panel). In comparison, the *nef*-defective forms consistently depleted CD4$^+$ T cells less efficiently. Predictably, the replication-incompetent *nef*+TPImut and *nef**TPImut HIV-1 variants did not cause cytopathic effects in ex vivo-infected HLT. We did also observe a minor reduction of CD4$^+$ T cells in HLT infected with the R5-tropic forms of NL4-3 expressing full-length Nef proteins. It has been previously shown that R5- and X4-tropic HIV-1 replicate with comparable efficiency and are equally cytopathic for their T cell targets in HLT (Grivel and Margolis, 1999). However, because only a small fraction of CD4$^+$ T cells in HLT expresses CCR5, only up to 20% were depleted (data not shown).

In sum, our results clearly demonstrate that HIV-1 lacking the modulatory U3 region and the *nef*-LTR overlap can replicate efficiently and cause CD4$^+$ T cell depletion in human T cells and ex vivo-infected HLT. Compared to NL4-3wt, however, the *nef*+TPImutΔ1Δ2 mutant virus showed delayed replication kinetic and attenuated cytopathic effects in some ex vivo-infected HLTs. In comparison, analogous SIV *nef*-LTR variants consistently replicated with 239wt-like kinetics in cell culture assays and in rhesus macaques (Münch et al., 2001). These data are in agreements with previous findings demonstrating that the SIVmac U3 region shows a higher degree of functional redundancy than that of HIV-1 (Pöhlmann et al., 1998) and indicate that the modulatory U3 region of HIV-1 contributes moderately to viral gene expression. Our results obtained in ex vivo-infected HLT suggest that HIV-1 variants with short U3 regions that do not overlap *nef* might be pathogenic in vivo, although they will most likely not induce disease as consistent as wild type HIV-1 strains. Why the modulatory HIV-1 U3 region contribute to optimal viral spread in cells or tissues from some donors remains to be clarified. Possible explanations might be donor to donor variations in the expression of specific transcription factors that bind to the upstream U3 sequences. Importantly, intact *nef* genes

consistently enhanced viral replication of the mutant viruses demonstrating that the HIV-1 constructs described in this study are useful to analyze Nef function without the restriction and complications of overlapping LTR and critical *cis*-regulatory elements.

Materials and methods

Mutant construction

Site-directed mutagenesis to generate the HIV-1 NL4-3 TPI variants was performed by spliced overlap extension (SOE) PCR as described previously (Münch et al., 2001). All PCR products were cloned in pCR2.1-Topo vector (Invitrogen) and sequenced on both strands. First a *Sma*I site was introduced in pBRNL4-3 just upstream of the PPT/Int sequences using pBamHI and pLsmaNRE (left half) and pRsmaNRE and pXbaI (right half). SOE PCR of gel purified fragments was performed with outer primers pBamHi and pXbaI, sequenced and cloned via *Bam*HI and *Xba*I into pBRNL4-3, resulting in the generation of pBRNL4-3(Δ1). Next, *env-nef* or *env-nef** fragments were generated using primer pairs pBamHI and pnef*SmaI with pBRNL4-3wt and NL4-3nef* as template. pnef*SmaI introduces a stop codon downstream of the *nef* ORF. PCR fragments were cloned into pBRNL4-3(Δ1) using *Bam*HI and *Sma*I resulting in the generation of pBRNL4-3 nef+Δ1 and nef*Δ1. nefPmutΔ1 was generated by SOE PCR using outer primers pBamHI and pnef*SmaI and PCR fragments as template obtained using pBamHI and pLPPNLneu (left fragment) and pRPPNL and pnef*Sma (right fragment). nefTPImutΔ1 was generated the same way except that mutagenic primers were p5′PPT/INTmut and p3′PPT/INTmut. PCR fragments were cloned in pBRNL4-3(Δ1) via *Bam*HI and *Sma*I. pBRNL4-3 nefPmut and nefTPImut were generated using the same strategy except that the outer primer pXbaI was used instead of pnef*SmaI. To generate pBRNL4-3 Δ1Δ2 PCR was performed with pBRNL4-3(Δ1) as template and primer pairs pBamHI and p5′ΔNRE (left fragment) and p3′ΔNRE and pXbaI (left fragment). SOE PCR with gel purified PCR products was performed using pBamHI and pXbaI and the resulting fragment was cloned into pBRNL4-3. NL4-3 nef+Δ1Δ2, nef*Δ1Δ2, nefPmutΔ1Δ2 and nefTPImutΔ1Δ2 were generated by cloning the corresponding mutants from Δ1 constructs into pBRNL4-3 Δ1Δ2 using *Bam*HI and *Sma*I. R5 tropic variants were generated by replacing the wild type nef/LTR fragment of pBRNL4-3 005pf135 (Papkalla et al., 2002) with the indicated nef/LTR mutants using *Hpa*I and *Xba*I. Primer sequences were: pRsmaNRE 5′-GAAAATTTTTCACCGAT*GGGCCC*TCTAGTCTAGATGTCGACGGAAC-3′; pLsmaNRE 5′-GTTCCGTCGACATCTAGA*GGGCCC*ATCGGTGAAAAATTTTC-3′; pRPPNL 5′-CACTTAATCGGGAAGGTCCGGTGGAAAGAGGAACTCCTTCACCGATTC-3′; pLPPNLneu 5′-CATCTAGAATCGGTGAAGGAGTTCCTCTTTCCACCGGACCTTCCC-3′; p5ΔNRE 5′-CTTGAAGTACTCCGGATGGGATATCTTGTCTTCTTT-3′; p3′ΔNRE 5′-CCAAAGAAGACAAGATATCCCATCCGGAGTACTTCAAG-3′; p5′PPT/INTmut 5′-ATCTAGAATCGGTGAAGGAGTTCCTCTTTCCACCGAATCTCCCAAAC-3′; p3′PPT/INTmut 5′-GAAACCCTCACTTAGTTTGGGAGATTCGGTGGAAAGAGGAACTCCTT-3′; pnef*Sma 5′-ATGAAGTTCTTGACGACTTGCGCA*GGGCCC*CCT-3′; pBamHI 5′-TAGTGAACGGATCCTTAGC-3′ and pXbaI 5′-GGTTGTCTAGAACTGCTAGAGATTTTCCACACT-3′.

Cells

CEM × 174 5.25 M7 cells, kindly provided by Nathaniel Landau, were kept in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). P4-CCR5 cells, provided through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (Charneau et al., 1994), and 293T cells were kept in DMEM medium supplemented with 10% FCS. Human PBMC were isolated by use of lymphocyte separation medium (Organon Teknika Corporation, Durham, N.C.) and either cultured in RPMI (10% FCS; unstimulated) or stimulated with RPMI (20% FCS) supplemented with 4 μg/ml phytohemagglutinin (Sigma) and 10 ng/ml IL-2 (Strathmann) for 3 days.

Production of virus stocks and infection experiments

Virus stocks were produced by transient transfection of 293T cells as described previously (Rucker et al., 2004). Briefly, 293T cells were transfected with plasmids containing the entire HIV-1 NL4-3 genome by using a CaPO technique. The culture medium was changed 1 day after transfection, and the culture supernatant was harvested the following day. Residual cells in the supernatants were pelleted, the supernatants were passed through 0.45-μm-pore-size filters and stored at −70 °C. The content of viral p24 antigen was quantified by an HIV p24 enzyme-linked immunosorbent assay kit obtained through the AIDS Research and Reference Reagent Program. P4-CCR5 cells (4000 cells per well) were sown out in 96 well dishes in a volume of 100 μl and infected after overnight incubation with virus stocks containing 3 ng of p24 antigen in a total volume of 200 μl. Three days post-infection viral infectivity was detected using the Gal Screen Kit (Tropix) as recommended by the manufacturer. To calculate percent values, relative light units per second (RLU/s) obtained for HIV-1 NL4-3 wild type infection were set to 100%. 2×10^5 unstimulated or stimulated PBMC were sown out in 150 μl medium and infected with virus stocks containing 1 ng p24 antigen in a total volume of 200 μl. Unstimulated PBMC were stimulated 3 days post-infection with PHA (3 μg/ml) and 10 ng/ml IL-2. Thereafter, all PBMC were cultivated in RPMI containing 10 ng/ml IL-2. Supernatants

were harvested at regular intervals and fresh medium was added. The extent of viral replication was determined by using a radioactive reverse transcription (RT) assay as previously described (Potts, 1990). All experiments were performed with at least four independent virus stocks in triplicate.

HIV infection of human lymphoid tissue ex vivo

Human tonsillar tissue removed during routine tonsillectomy and not required for clinical purposes was received within 5 h of excision. The tonsils were washed thoroughly with medium containing antibiotics and then sectioned into 2- to 3-mm blocks. These tissue blocks were placed on top of collagen sponge gels in the culture medium at the air–liquid interface and infected as described previously (Glushakova et al., 1995, 1997). In a typical experiment, 3 to 5 μl of clarified virus containing medium was applied to the top of each tissue block. Supernatants were collected at 3 day intervals and productive HIV infection was assessed by measuring p24 antigen content. Flow cytometry was performed on cells mechanically isolated from control and infected tissue blocks and depletion of $CD4^+$ T cells was assessed as described previously (Glushakova et al., 1997).

Genomic DNA preparation and PCR analysis

2×10^6 CEM × 174 5.25 M7 were sown out in 300 μl medium in 48 well dishes and infected in a total volume of 500 μl with 10 ng of p24 antigen. 4 days post-infection fresh cells were added. 10 dpi cells were pelleted, washed in PBS, lysed in 100 μl cell lysis buffer (50 mM KCl, 10 mM Tris pH 8.3, 2.5 mM MgCl2, 0.45% NP-40, 0.45% Tween-20 and 1 mg/ml proteinase K) and incubated for 1 h at 56 °C. After proteinase inactivation for 10 min at 95 °C, 5 μl of lysates were used for PCR analysis using oligonucleotides p1 5′-GGCTACTTCCCTGATTGGCAGAA-3′, p2 5′-CCGTGCGCGCT-TCAGCAAGCCGAG-3, p4 5′-GGGTCTGAGGGATCTCTAGTTACC-3 and p5 5′-GGATATCTTGTCTT-CTTTGGGAGTGA-3.

Western blot analysis

293T cells were transiently transfected in T-25 flasks, the medium was changed 12 to 16 h after transfection and cells were harvested 48 h after transfection. The cells were rinsed with phosphate-buffered saline and lysed in RIPA-1 buffer (1% Triton X-100; 0.15 M NaCl; 50 mM Tris–HCl pH 7.4; 5 mM EDTA). Immunoblots were performed using rabbit-anti-HIV-p24 serum provided by the AIDS Research and Reference Reagent Program or an anti-HIV-1 Nef mAb directed against amino acids 151 to 170 (Advanced Biotechnologies). As a positive control, cells were transfected with the pCG-NL4-3Nef vector, which contains the CMV promoter and coexpresses Nef and the green fluorescent protein.

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